

Cultivar-specific avirulence and virulence functions assigned to *avrPphF* in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease

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The *avrPphF* gene was cloned from *Pseudomonas syringae* pathovar *phaseolicola* (*Pph*) races 5 and 7, based on its ability to confer avirulence towards bean cultivars carrying the *R1* gene for halo-blight resistance, such as Red Mexican. *avrPphF* comprised two open reading frames, which were both required for function, and was located on a 154 kb plasmid (pAV511) in *Pph*. Strain RW60 of *Pph*, lacking pAV511, displayed a loss in virulence to a range of previously susceptible cultivars such as Tendergreen and Canadian Wonder. In Tendergreen virulence was restored to RW60 by *avrPphF* alone, whereas subcloned *avrPphF* in the absence of pAV511 greatly accelerated the hypersensitive resistance reaction caused by RW60 in Canadian Wonder. A second gene from pAV511, *avrPphC*, which controls avirulence to soybean, was found to block the activity of *avrPphF* in Canadian Wonder, but not in Red Mexican. *avrPphF* also conferred virulence in soybean. The multiple functions of *avrPphF* illustrate how effector proteins from plant pathogens have evolved to be recognized by *R* gene products and, therefore, be classified as encoded by avirulence genes.

Keywords: avirulence/disease resistance/gene-for-gene interactions/hypersensitive reaction/virulence

Introduction

The bacteria and fungi that colonize living plants and cause disease have evolved the ability to overcome the innate resistance of their hosts to infection. Superimposed on this establishment of basic parasitism (or pathogenicity) is the phenomenon of cultivar (cv.)-specific resistance. A plant pathogen may, therefore, be fully virulent causing disease on certain cultivars of its host, but avirulent on others (De Wit, 1995; Alfano and Collmer, 1997; Grant and Mansfield, 1999). Avirulence is associ-

ated with activation of the hypersensitive reaction (HR), a form of rapid programmed cell death, which restricts colonization to the site of inoculation in resistant cultivars of the host plant (De Wit, 1995; Mansfield *et al.*, 1997a). This article concerns the genetics of avirulence and virulence in the bean halo-blight bacterium *Pseudomonas syringae* pathovar (pv.) *phaseolicola* (hereafter *Pph*). Nine races of *Pph* have been differentiated based on their virulence to a range of bean cultivars, as summarized in Table I (Taylor *et al.*, 1996).

The gene-for-gene theory explaining cultivar-specific resistance to plant disease was developed by Flor (1942, 1971) based on his analysis of the differential reactions of flax to the rust fungus *Melampsora lini*. Person *et al.* (1962) summarized the essence of Flor's hypothesis in the statement that '...for every resistance gene in flax there is a specific and related gene for virulence in those rusts to which it is susceptible'. The emphasis on susceptibility suggested that disease was the result of the positive function of cultivar-specific virulence determinants. The advances of molecular genetics have proved the gene-for-gene theory by the cloning not of virulence (*vir*) genes, but of avirulence (*avr*) genes from bacteria and fungi, and the matching resistance (*R*) genes from plant hosts (Dangl, 1994; De Wit, 1995; Jones and Jones, 1996; Vivian *et al.*, 1997). The proteins encoded by *avr* and *R* genes are envisaged to interact either directly or indirectly to activate signalling cascades leading to resistance, which is expressed by the HR (Hammond-Kosack and Jones, 1996; Van den Ackerveken *et al.*, 1996). Bacterial *avr* genes have been cloned by function through the transfer of genomic libraries from avirulent to virulent races of the pathogen (Staskawicz *et al.*, 1987). Genomic clones causing changes in the virulence phenotype on certain cultivars have been isolated and genes for avirulence subsequently characterized, e.g. *avrPphB* and *avrPphE* from *Pph*, which correspond to *A3* and *A2*, respectively, in Table I (Mansfield *et al.*, 1994; Vivian *et al.*, 1997). Similarly, genes for avirulence but not virulence have also been cloned, which operate at the level of host specificity (Kobayashi *et al.*, 1989; Fillingham *et al.*, 1992; Wood *et al.*, 1994).

Clearly, the continued presence of genes that restrict host range in certain strains or races of plant pathogens is somewhat of a paradox, but some *avr* genes have been implicated in basic parasitism, e.g. *avrRpm1* from *P.s.* pv. *maculicola* and *avrE* from *P.s.* pv. *tomato*. Mutations in *avrRpm1* or *avrE* lead to greatly reduced ability to colonize host plants (Lorang *et al.*, 1994; Ritter and Dangl, 1995). The emerging pattern is that plant pathogens may produce numerous proteinaceous virulence factors, which act synergistically to promote disease (Alfano and Collmer, 1997; Collmer, 1998). Some of these factors may become recognized by the products of *R* genes and,

Table I. Gene-for-gene relationship (based on five matching gene pairs) proposed from analysis of the reactions^a of bean cultivars to races of *P.s. pv. phaseolicola* (from Taylor *et al.*, 1996)

	Races/avirulence (A) genes													
	1	2	3	4	5	6	7	8	9					
	<i>A1</i>	.	.	.	<i>A1</i>	.	<i>A1</i>	.	<i>A1</i>	.	<i>A1</i>	.	<i>A1</i>	.
	.	<i>A2</i>	.	<i>A2</i>	<i>A2</i>	.	<i>A2</i>	.	<i>A2</i>	.	<i>A2</i>	.	<i>A2</i>	.
	.	.	<i>A3</i>	<i>A3</i>
	<i>A4</i>
Differential cultivars	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>	<i>A5</i>
Resistance genes														
Canadian Wonder
A52 (ZAA54)	.	.	.	<i>R4</i>
Tendergreen	.	.	<i>R3</i>
Red Mexican	<i>R1</i>	.	.	<i>R4</i>
A53 (ZAA55)	.	.	<i>R3</i>	<i>R4</i>
A43 (ZAA12)	.	<i>R2</i>	<i>R3</i>	<i>R4</i>	<i>R5</i>

^a+, susceptible; –, resistant.

therefore, be identified as determinants of avirulence. Because of functional redundancy, however, the loss of what has become an *avr* gene rather than a *vir* gene does not always compromise the basic pathogenicity on which cultivar specificity is superimposed.

The *vir* gene concept has been strengthened by the identification of a gene, *virPphA* from *Pph*, based on its ability to restore virulence to strains of the bean pathogen cured of a 154 kb plasmid designated pAV511 (Jackson *et al.*, 1999). Cured strains (such as that named RW60) lost virulence towards bean, causing hypersensitive reactions in all previously susceptible cultivars tested. A cluster of potential *vir* genes including *virPphA* was located to a region described as a pathogenicity island (PAI), on pAV511. The ability of RW60 to elicit the HR suggested that it contained *avr* genes, located on the chromosome or other plasmids, whose function was usually masked by virulence factors encoded by the PAI. Other *avr* genes, such as *avrPphB* or *avrPphE*, continued to function despite the operation of the plasmid-borne PAI in wild-type strains and were, therefore, classified as α *avr* genes. The genes detected only in the absence of the PAI were classified as β *avr* genes (Jackson *et al.*, 1999).

The resistance responses activated by different *avr*–*R* gene combinations may all be classified as the HR, but they may be phenotypically different because of, for example, differential timing of plant cell collapse. Differences are clear in bean as the *avrPphB*–*R3* interaction provokes a more rapid HR than *avrPphE*–*R2* (Mansfield *et al.*, 1994, 1997a,b). In many race–cultivar interactions, several different α *avr* genes and their matching *R* genes may be present in the pathogen and plant, respectively (Mansfield *et al.*, 1997a). The potential for epistatic interaction between various *avr*–*R* gene combinations is apparent from analysis of *Arabidopsis* harbouring the *RPM1* and *RPS2* genes. Although the HR controlled by *avrRpt2*–*RPS2* is slower than that activated by the *avrRpm1*–*RPM1* interaction, the phenotypically slower response is observed when both *avr* genes are present (Reuber and Ausubel, 1996; Ritter and Dangl, 1996). It has been proposed that such epistasis involves direct interaction between *avr* and *R* gene products (Ritter and Dangl, 1996).

Here we describe molecular characterization of the *avrPphF* locus in *Pph*. The gene was found to comprise an operon with two open reading frames (ORFs), both of which were required for function. Although we identified *avrPphF* as an α *avr* gene that determined race structure in the halo-blight bacterium, it was also found to confer cultivar-specific virulence to cv. Tendergreen. Furthermore, in the absence of the plasmid pAV511, containing the PAI, *avrPphF* also acted as a β *avr* gene in cv. Canadian Wonder, which is susceptible to all known races of the halo-blight bacterium. The gene within pAV511 that masked the β type of *avr* activity of *avrPphF* to Canadian Wonder was found to be *avrPphC*, which was previously identified as an *avr* gene acting on soybean cultivars. In short, an intriguing web of interacting avirulence and virulence functions has been revealed for the *avr* genes from *Pph*. In the bean–*P.syringae* interaction, the gene-for-gene concept, although continuing to provide a useful framework for analysis of cultivar-specific resistance, is far more complex than is apparent from simple analysis of race structures and α *avr*–*R* gene interactions as outlined in Table I. It is proposed that similar complexity will emerge in other host–pathogen systems once the problem of the redundancy of multiple virulence factors has been overcome by the removal of certain PAIs.

Results

Cloning of avrPphF from race 5

Based on the race structure proposed in Table I (Taylor *et al.*, 1996), we sought to clone the putative *A1* and *A4* genes from race 5. A genomic library of race 5 strain 1375A, predicted to contain *A1* and *A4* and known to harbour *A2* (cloned as *avrPphE* by Stevens *et al.*, 1998), was screened for *avr* genes by mating into race 6 strain 1448A, which is virulent on all cultivars of bean used to define race structure (Mansfield *et al.*, 1994). Transconjugants were initially tested for virulence on pods of cvs Red Mexican UI3 (proposed *R1*, *R4*) and A53 (*R3*, *R4*). Two genomic clones, pPPY501 and pPPY502, containing overlapping regions of insert DNA conferred avirulence on Red Mexican but none from the 1500 tested

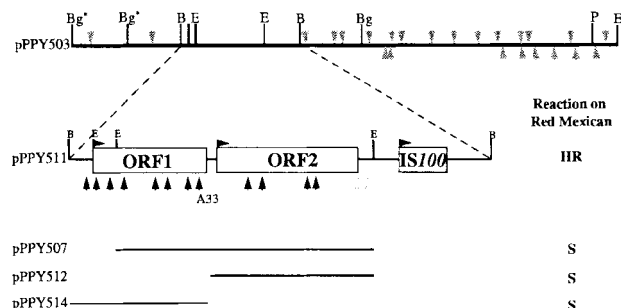


Fig. 1. Location of *avrPphF* within pPPY503 and the 1.8 kb of DNA sequenced. The direction of transcription is indicated by the horizontal arrowheads. Sites of insertion of *Tn3gus* in pPPY503 are indicated by vertical arrowheads; only those marked black in the expanded fragment abolished the avirulence activity of *avrPphF*. All transposon insertions were mapped from restriction digests, except A33, which was located by sequencing. The region found to have similarity to *IS100* is indicated. Restriction sites for *Bam*HI (B), *Bgl*III (Bg), *Eco*RI (E) and *Pst*I (P) are marked; an asterisk indicates site in the vector pLAFR3. The reactions caused in bean cv. Red Mexican by transconjugants of race 6 harbouring subclones are noted. HR, hypersensitive reaction; S, susceptible response.

affected reactions on A53. Avirulence activity was retained by a 5.3 kb fragment, designated pPPY503. Transposon mutagenesis with *Tn3gus* located *avrPphF* to a 1.3 kb region flanked by *Bam*HI and *Eco*RI restriction sites (Figure 1). The 1.8 kb *Bam*HI fragment containing this region was cloned as pPPY511, and found to activate the HR in a cultivar-specific manner; *avrPphF* therefore acted as a typical α *avr* gene.

Segregation of the *R1* resistance gene matching *avrPphF* was examined in 88 F_2 progeny derived from a cross between Red Mexican and Tendergreen (*R3*). Progeny undergoing the HR to the transconjugant race 6 (pPPY503) were also resistant to race 1 (*A1*). The pattern of incompatibility observed fitted that expected if *avrPphF* matched the *R1* gene in Red Mexican, the numbers of resistant and susceptible plants (66:22) exactly fitting the 3:1 ratio expected if the *R1* gene was dominant. The F_2 progeny from the cross between Red Mexican and Tendergreen were also tested with a transconjugant of race 6 containing the clone pMS2330, which contains *avrPphA* isolated by Shintaku *et al.* (1989). Although race 6 (pMS2330) did cause a weak HR on Red Mexican, reactions on pods from the F_2 progeny of Red Mexican and Tendergreen showed that there was no co-segregation with race 1 (data not shown).

Multiplication of race 1 and race 6 was compared with race 6 (pPPY503) in leaves of cv. Red Mexican. The HR determined by the presence of the cloned *avrPphF* gene was associated with the failure of bacteria to multiply. Following inoculation to introduce $\sim 0.2 \times 10^6$ c.f.u. per excised leaf disc, after 2 days race 6 had reached 3.7×10^6 , whereas race 1 and race 6 (pPPY503) were present at only 9.3×10^2 and 16×10^2 c.f.u. per disc, respectively.

Sequence analysis and expression of *avrPphF*

The nucleotide sequences of the functional 1.8 kb fragments from race 5, and as also recovered from race 7 containing *avrPphF*, were found to be identical (DDBJ/

EMBL/GenBank accession Nos AF231452 and AF231453). The sequence revealed two ORFs with a single upstream promoter region containing the *hrp* box motif, indicating probable regulation by HrpL (Innes *et al.*, 1993; Pirhonen *et al.*, 1996). Both ORFs were preceded by purine-rich regions, which would be expected to act as ribosome binding sites (Singer and Berg, 1991) and they were separated by a 57 bp sequence. A rho-dependent transcription termination loop was located after the translation stop signal in ORF2. The *avrPphF* locus therefore had the structural features of an operon. The G+C content of *avrPphF* was 47%, with ORF1 40% and ORF2 52.5%, both significantly lower than the overall figures of 59–61% reported for pvs of *P. syringae* (De Ley, 1968).

Transposon insertions that compromised avirulence were located in both ORF1 and ORF2 of *avrPphF*, and further subcloning revealed that both ORFs were required for *avr* function (Figure 1). To examine further the role of the two ORFs, non-polar mutations were created for ORF1 and ORF2, resulting in race 7 Δ ORF1 and race 7 Δ ORF2, respectively; both mutants failed to elicit the HR in cv. Red Mexican.

Protein production from ORFs 1 and 2 was examined in *Escherichia coli* using constructs of *avrPphF* cloned for expression in pBluescript. Proteins of 17 and 24 kDa were detected on SDS-PAGE gels after Coomassie Blue staining, corresponding to the peptides predicted to be encoded by ORFs 1 and 2, i.e. 15.6 and 21.9 kDa, respectively (data not shown). Searches of the databases using the BLAST or Propsearch approaches (Hobohm and Sander, 1995; Altschul *et al.*, 1997) failed to reveal significant homology with known protein sequences. Both peptides are predicted to be hydrophilic; they lack leader sequences or potentially membrane-spanning domains.

avrPphF is plasmid borne and present only in races expressing the *A1* phenotype

The presence of homologues of *avrPphF* was examined in strains of *Pph* representing all nine races and also in other pathogens of *P. syringae*. Using specific probes for ORF1 and ORF2 in PCR or Southern analysis, signals identical to those found in race 5, the origin of the *avrPphF* clone, were present only in races 1, 7 and 9, but not in races 2, 3, 4, 6 or 8, which are virulent on Red Mexican (Table I; Figure 2A and B). However, when the entire 1.8 kb *Bam*HI fragment containing *avrPphF* was used as a probe the pattern changed. Multiple signals were detected in all races of *Pph* (Figure 2C). Further probing revealed that multiple signals were due to the 0.5 kb *Eco*RI-*Bam*HI fragment downstream of *avrPphF* (Figure 1), which was found to contain an incomplete ORF with predicted similarity to the *IS100* transposase homologue from the PAI in *Pph* (Jackson *et al.*, 1999). Sequences hybridizing to *avrPphF* were found in strains of *P.s.* pvs *pisi* and *glycinea*, but not in pvs *coronafaciens*, *maculicola*, *tabaci*, *tomato* or *syringae*, or in *Pseudomonas cichorii*. Interestingly, only *P.s.* pv. *syringae* and *P.s.* pv. *pisi* contained sequences hybridizing to the putative transposase, but, in contrast to *Pph*, only one band was observed on Southern blots (data not shown), indicating that it is highly specific to pv. *phaseolicola*.

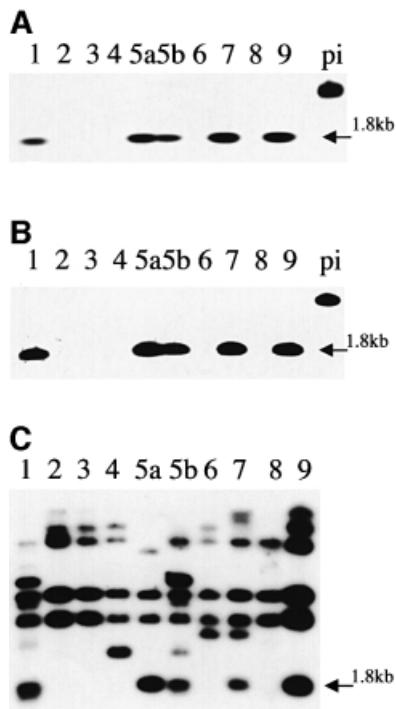


Fig. 2. Southern hybridization (high stringency) of *Bam*HI-digested total DNA from different strains of *P. syringae* using (A) ORF1 and (B) ORF2 of *avrPphF* and (C) the 0.5 kb *Eco*RI-*Bam*HI fragment with similarity to *IS100* as probes. Digests from races 1, 2, 3, 4, 5 (a, strain 52A; b, strain 1375A), 6, 7, 8 and 9 of *P. s. pv. phaseolicola*, and *P. s. pv. pisi* (pi) are shown.

Hybridization experiments also located *avrPphF* to the 154 kb indigenous plasmid pAV511. *avrPphF* was positioned at the left of the PAI recently identified in *Pph* (Figure 3), close to avirulence genes *avrPphC* and a homologue of *avrD*, both of which were cloned as soybean interactors (Kobayashi *et al.*, 1990; Yucel *et al.*, 1994).

***avrPphF* acts as a gene for virulence in cv. Tendergreen**

Although race 7 of *Pph* is virulent on cv. Tendergreen, the plasmid-cured strain RW60 causes an HR. Virulence towards Tendergreen was restored by the genomic clones pAV520 or pAV521 harbouring *avrPphF* recovered from race 7 strain 1449B (Jackson *et al.*, 1999). A transposon insertion in pAV521, which reduced ability to restore virulence only to cv. Tendergreen, was located to *avrPphF* and we subsequently found that *avrPphF* alone (pPPY511) effectively restored water-soaking ability to RW60 in pods and leaves of Tendergreen, as well as the distinct *avrPphF*-*R1* interaction phenotype of the HR in cv. Red Mexican (Table II; Figure 4A). The complementation achieved showed, therefore, that *avrPphF* acts as a cultivar-specific virulence gene on cv. Tendergreen. Transconjugants, RW60(pPPY514) and RW60 (pPPY512), separately harbouring ORF1 and ORF2 of *avrPphF*, respectively, were unable to restore virulence (Table II).

The increased virulence of transconjugants of RW60 harbouring *avrPphF* was also demonstrated by increases in bacterial populations in Tendergreen leaves (Figure 5). Interestingly, bacterial growth of the marker exchange *avrPphF* mutant, race 7:*avrPphF* Tn3gus A33 (Figure 1), was restricted compared with the wild type, but the

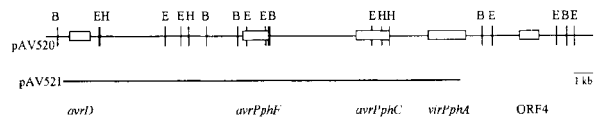


Fig. 3. Location of *avrPphF* in relation to *avrD*, *virPphA*, *avrPphC* and ORF4 in the 154 kb plasmid identified in race 7 strain 1449B (Jackson *et al.*, 1999). The insert cloned in pAV520 (vector pLAFR3) is shown; the region present in pAV521 is underlined. Restriction sites for *Bam*HI (B), *Eco*RI (E) and *Hind*III (H) are marked.

symptoms produced were still classified as susceptible. The two non-polar mutants, race 7 Δ ORF1 and race 7 Δ ORF2, respectively, were also virulent in cv. Tendergreen. The continued presence of plasmid-borne *virPphA*, and possibly other *vir* genes in race 7 strains, may allow the *avrPphF* mutants to grow but not to reach wild-type levels.

***avrPphF* behaves as a masked avirulence gene in cv. Canadian Wonder**

The plasmid-cured strain RW60 was found to cause a slow HR (subsequently designated hr) in cv. Canadian Wonder, whereas wild-type race 7 causes water soaking characteristic of a susceptible reaction. Unexpectedly, transconjugant RW60(pPPY511), expressing *avrPphF*, caused a very rapid HR on leaves and pods of cv. Canadian Wonder quite distinct from the hr caused by RW60 alone (Figure 4B). Transconjugants of RW60 containing pAV520 (which harbours *avrPphF*; Figure 3), like wild-type race 7, caused a susceptible reaction on cv. Canadian Wonder, indicating that a gene in pAV520 must suppress the avirulence function of *avrPphF*. Analysis of subcloned regions of pAV520 revealed that the gene suppressing the *avr* activity of *avrPphF* was the non-host avirulence gene *avrPphC* (Yucel *et al.*, 1994). Transconjugants of RW60 containing both *avrPphF* and *avrPphC* (in pPPY511 and pDAHR15, respectively) gave a phenotype identical to that caused by RW60 (Table II; Figure 4B). Multiplication of race 7, RW60, RW60(pPPY511) and RW60(pPPY511, pDAHR15) in leaves was compared. The rapid HR determined by the presence of *avrPphF* in RW60(pPPY511) was associated with the recovery of very low numbers of bacteria 96 h after inoculation, whereas RW60(pPPY511, pDAHR15) achieved bacterial numbers similar to RW60 alone, confirming the phenotypes observed (Figure 6).

Analysis of segregating plant populations

The phenotypes caused by RW60, RW60(*avrPphF*) and RW60(*avrPphF*, *avrPphC*) were very distinct, as shown in Figure 4A and B. We were, therefore, able to analyse the segregation of reactions in crosses between cvs Canadian Wonder and Tendergreen. Analyses of F₂ populations summarized in Tables III and IV are based on the following proposals: (i) Canadian Wonder has two *R* genes, *RF* matching *avrPphF* and *R β 2* matching a β *avr* gene unmasked in RW60; (ii) Tendergreen has *R β 1* matching a second β *avr* gene in RW60, but does not harbour *RF*; (iii) the plasmid-cured strain RW60 causes a rapid HR on cv. Tendergreen and hr on cv. Canadian Wonder due to the *avr β 1*-*R β 1* and *avr β 2*-*R β 2* interactions, respectively; (iv) *avrPphF* acts as a virulence

Table II. Reactions^a caused by strains of *P.s. pv. phaseolicola* on bean and soybean cultivars

Strain ^b	Bean cultivar			Soybean cultivar	
	Red Mexican	Tendergreen	Canadian Wonder	Osumi	Choska
Race 7	HR*	S	S	HR	S ⁻
RW60	HR	HR	hr	N	N
RW60(pAV520)	HR*	S	S	HR	S ⁻
RW60(pPPY511)	HR*	S ⁻	HR	S ⁻	S ⁻
RW60(pPPY512)	HR	HR	hr	N	N
RW60(pPPY514)	HR	HR	hr	N	N
RW60(pPPY511, pDAHR15)	HR*	S ⁻	hr	nt	nt
Race 7:: <i>avrPphFTn3gus</i> A33	S	S	S	nt	nt
<i>P.s. pv. glycinea</i>	HR	HR	HR	S	S

^aS, fully susceptible water-soaked lesion; S⁻, slower development of lesions than S; HR, hypersensitive reaction; HR*, characteristic of the *avrPphF*-*R1* interaction; hr, slow development of the HR; N, null reaction; nt, not tested.

^bpAV520 is a genomic clone; plasmids encoding single ORFs or *avr* genes are pPPY511 (*avrPphF*), pPPY512 (*avrPphF*, ORF2), pPPY514 (*avrPphF*, ORF1) and pDAHR15 (*avrPphC*).

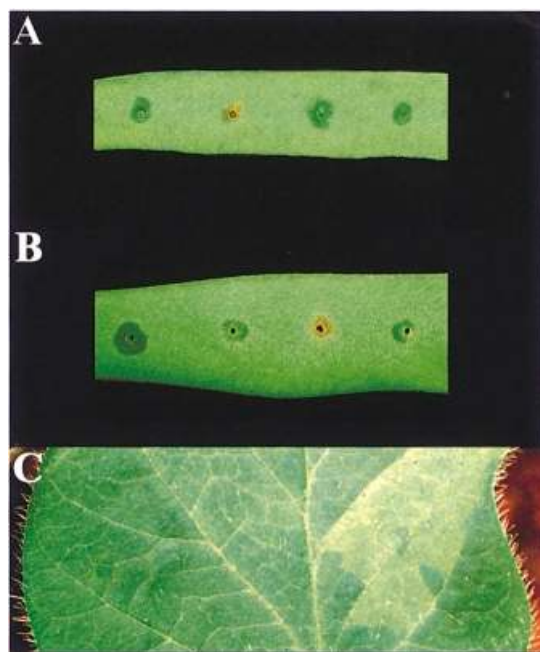


Fig. 4. (A) Reaction phenotypes in a pod of cv. Tendergreen 2 days after inoculation with (from left to right) race 7, RW60, RW60 (pAV520) containing the PAI and RW60(pPPY511) expressing *avrPphF* alone. (B) Reaction phenotypes in a pod of cv. Canadian Wonder 2 days after inoculation with (from left to right) race 7, RW60, RW60(pPPY511) expressing *avrPphF*, and RW60(pPPY511, pDAHR15) expressing both *avrPphF* and *avrPphC*. Note that the lesions caused by RW60 and RW60(pPPY511, pDAHR15) are sunken at this stage compared with the water-soaked susceptible response to race 7, whereas RW60(pPPY511) has already caused a brown lesion characteristic of the rapid HR induced. (C) Reactions at infiltration sites in soybean leaves (cv. Osumi) 3 days after inoculation with (left to right) RW60, which causes a null reaction, and the transconjugant RW60(pPPY511) expressing *avrPphF*, which causes a susceptible response recognized by yellowing as shown and later some water-soaking.

determinant on cv. Tendergreen. The phenotypes observed corresponded to those expected from these proposals, the parental genotypes predicted being *Rβ1*, *Rβ1*; *rβ2*, *rβ2*; *rF*, *rF* for Tendergreen, and *rβ1*, *rβ1*; *Rβ2*, *Rβ2*; *RF*, *RF* for Canadian Wonder.

The reaction of F₂ progeny to RW60 (Table III) indicated that the *avrβ2* gene, although conferring a

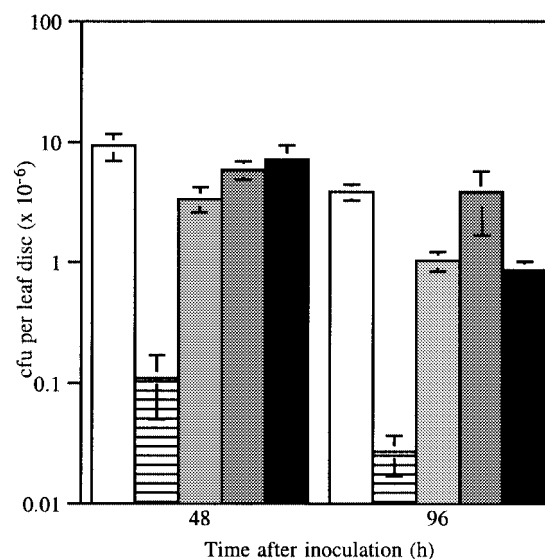


Fig. 5. Bacterial multiplication in leaves of bean cv. Tendergreen inoculated with suspensions of 2×10^8 cells/ml of race 7 (open), RW60 (striped), RW60 (pPPY511) (light grey), RW60 (pAV520) (dark grey) and the *avrPphF* marker exchange mutant race 7::*avrPphF* Tn3gusA33 (black); bars, \pm SEM.

slower hr, was epistatic to *avrβ1*. Phenotypes segregated hr:HR 31:7, closely approximating the predicted 13:3 ratio ($\chi^2 = 0.0025$). Reaction to RW60(*avrPphF*) clearly indicated the presence of the matching dominant *RF* gene in Canadian Wonder with phenotypes observed, HR:hr or S, 27:11 (Table IV). Amongst the 38 F₂ progeny examined, two plants developed a rapid HR when challenged by RW60 (indicating *avrβ1*-*Rβ1* interaction), but were susceptible to RW60(*avrPphF*), indicating the absence of *RF*. The virulence function of *avrPphF* therefore appeared to be directly related to blocking the proposed *avrβ1*-*Rβ1* interaction.

Nineteen of the 38 progeny were also tested with the RW60(*avrPphF*, *avrPphC*) transconjugant. Amongst the 64 possible genotypes predicted from the Canadian Wonder \times Tendergreen cross only three, *rβ1*, *rβ1*; *rβ2*, *rβ2*; *RF*, *RF* and *rβ1*, *rβ1*; *rβ2*, *rβ2*; *RF*, *rF* (or *rF*, *RF*) would be expected to produce a rapid HR when challenged by RW60(*avrPphF*) but be susceptible to RW60(*avrPphF*),

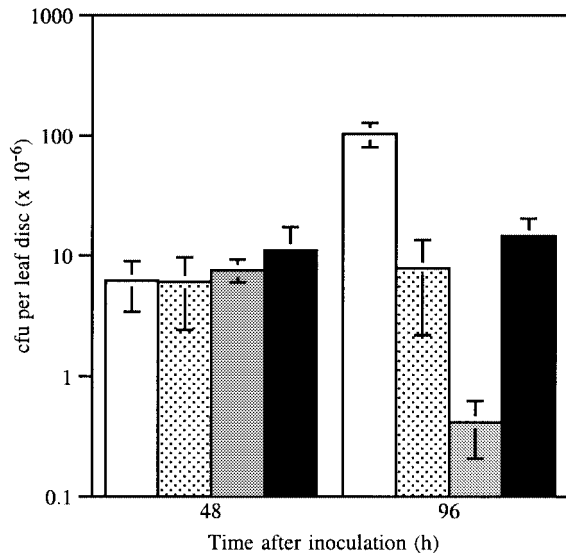


Fig. 6. Bacterial multiplication in leaves of bean cv. Canadian Wonder inoculated with bacterial suspensions of 2×10^8 cells/ml of race 7 (open), RW60 (stippled), RW60 (pPPY511) (grey) and RW60(pPPY511, pDAHR15) (black); bars, \pm SEM.

avrPphC). One plant was indeed detected with this phenotype, clearly supporting the gene-specific virulence function of *avrPphC* masking the *avrPphF*-*RF* interaction, and also the probable absence of other *R* genes, which would have prevented the establishment of a susceptible response.

***avrPphF* acts as a virulence gene in soybean**

Race 7 of *Pph* typically causes a rapid HR in soybean leaves, but in certain cvs such as Choska it was found to cause a weak susceptible response recognized by yellowing and water soaking at inoculation sites in leaves. The plasmid-cured strain RW60 caused a null response on all cvs of soybean tested. *avrPphF* was able to restore virulence to RW60 in cvs Choska and Osumi (Figure 4C; Table II). Restoration of virulence by *avrPphF* was also demonstrated by increases in bacterial populations in leaves of cv. Osumi (Figure 7).

Discussion

The pattern of avirulence conferred by *avrPphF* in wild-type races of *Pph* fits that predicted for an α *avr* gene

Table III. Segregation of resistance to RW60(*avrβ1*, *avrβ2*) amongst the F₂ progeny of a cross between Tendergreen (*Rβ1*, *Rβ1*; *rβ2*, *rβ2*)^a and Canadian Wonder (*rβ1*, *rβ1*; *Rβ2*, *Rβ2*)^a

Predicted genotype	Phenotype of response if <i>Rβ2</i> epistatic	Number of plants	
		Observed	Expected (13:3)
<i>Rβ1</i> , <i>Rβ1</i> ; <i>Rβ2</i> , <i>Rβ2</i> <i>rβ1</i> , <i>Rβ1</i> ; <i>Rβ2</i> , <i>Rβ2</i> <i>Rβ1</i> , <i>Rβ1</i> ; <i>Rβ2</i> , <i>rβ2</i> <i>rβ1</i> , <i>Rβ1</i> ; <i>Rβ2</i> , <i>Rβ2</i> <i>rβ1</i> , <i>rβ1</i> ; <i>Rβ2</i> , <i>Rβ2</i> <i>rβ1</i> , <i>Rβ1</i> ; <i>Rβ2</i> , <i>rβ2</i> <i>rβ1</i> , <i>rβ1</i> ; <i>Rβ2</i> , <i>rβ2</i> <i>rβ1</i> , <i>rβ1</i> ; <i>Rβ2</i> , <i>rβ2</i> <i>rβ1</i> , <i>rβ1</i> ; <i>rβ2</i> , <i>rβ2</i> ^b	Slow hr or susceptible	31 0	30.9
<i>Rβ1</i> , <i>Rβ1</i> ; <i>rβ2</i> , <i>rβ2</i> <i>rβ1</i> , <i>Rβ1</i> ; <i>rβ2</i> , <i>rβ2</i>	Rapid HR	7	7.1

^a*Rβ1* confers a rapid HR and *Rβ2* a slow hr.

^bThe predicted phenotype would be a susceptible reaction, unless there are other β *avr* genes in RW60.

Table IV. Segregation of resistance to RW60 (*avrβ1*, *avrβ2*, *avrPphF*^a) amongst the F₂ progeny of a cross between Tendergreen (*Rβ1*, *Rβ1*; *rβ2*, *rβ2*; *rF*, *rF*) and Canadian Wonder (*rβ1*, *rβ1*; *Rβ2*, *Rβ2*; *RF*, *RF*)

Predicted genotypes	Frequency	Phenotype	Number of plants	
			Observed	Expected (3:1)
Dominant ^b <i>Rβ1</i> , Dominant <i>Rβ2</i> , Dominant <i>RF</i> Dominant <i>Rβ1</i> , recessive <i>rβ2</i> , Dominant <i>RF</i> recessive <i>rβ1</i> , Dominant <i>Rβ2</i> , Dominant <i>RF</i> recessive <i>rβ1</i> , recessive <i>rβ2</i> , Dominant <i>RF</i> Total	27 9 9 3 48	Rapid HR	27	28.5
Dominant <i>Rβ1</i> , Dominant <i>Rβ2</i> , recessive <i>rF</i> Dominant <i>Rβ1</i> , recessive <i>rβ2</i> , recessive <i>rF</i> ^c recessive <i>rβ1</i> , Dominant <i>Rβ2</i> , recessive <i>rF</i> recessive <i>rβ1</i> , recessive <i>rβ2</i> , recessive <i>rF</i> ^c Total	9 3 3 1 16	Slow hr or Susceptible	9 2	9.5

^a*avrPphF* present on pPPY511. The parental phenotypes were rapid HR and susceptibility on cvs Canadian Wonder and Tendergreen, respectively.

^bDominant means homozygous (*RR*) or heterozygous (*Rr*).

^cPredicted to develop a susceptible reaction due to suppression of *Avrβ1* by *AvrPphF* or with all *R* genes recessive, if no other *avr*-*R* gene interactions occurring.

matching the *R1* gene for resistance in *Phaseolus* (Taylor *et al.*, 1996). The cloning strategy adopted failed to isolate the fourth *avr* gene predicted to match *R4* (Table I), possibly because the gene may have lethal effects in *E.coli*. The first *avr* gene cloned from *Pph* (Shintaku *et al.*, 1989), and designated *avrPphA* according to the nomenclature proposed by Vivian and Mansfield (1993), did not appear to match any of the *R* genes recognized in *Phaseolus*. The proposed gene-for-gene pattern of race structure (Taylor *et al.*, 1996) has, therefore, not been entirely confirmed by molecular genetics.

The *avrPphF* locus is organized into an operon with a characteristic '*hrp* box' promoter indicating regulation by HrpL. Both ORFs within the operon were required for either avirulence or virulence functions. The requirement for more than one transcriptional unit for the function of an *avr* gene was described in detail for *avrE* from *P.s.* pv. *tomato* by Lorang and Keen (1995). The *avrE* locus is linked to the right end of the *hrp* region and comprises two convergently transcribed units, which are both required for the avirulence phenotype. *avrBs1* from *Xanthomonas campestris* pv. *vesicatoria* comprises an operon of two

ORFs, but only the second is required for avirulence activity (Ronald and Staskawicz, 1988). Here, we show that *avrPphF* is organized into an operon with two ORFs, both of which are needed for function.

The mechanism by which *avrPphF* causes effects in plant cells remains unknown. According to published reports, all of the bacterial *avr* genes that have been tested elicit the HR if they are expressed transiently in plant cells; examples are *avrPphB* and *avrPphE* from *Pph* (Stevens *et al.*, 1998). The implication from *in planta* expression is that the Avr proteins act as elicitors following their delivery into plant cells by the *hrp*-dependent type III secretion apparatus (Alfano and Collmer, 1997; Bonas and Van den Ackerveken, 1997; Galan and Collmer, 1999). *In planta* expression of the ORFs from *avrPphF* should reveal whether one functions as the elicitor *per se* and one perhaps acts as a chaperone for protein delivery. The two ORFs were always found together, arranged into the *avrPphF* operon, but only in strains expressing the A1 phenotype (Table I; Figure 2). The adjacent *IS100* homologue was distributed throughout strains irrespective of the race designation. *IS100* is present in species of *Yersinia* and several copies have been located in plasmids encoding type III secretion systems or other virulence factors (Buchrieser *et al.*, 1998; Hu *et al.*, 1998). Sequences hybridizing to the insertion sequence were, however, not widely distributed amongst other pathovars of *P.syringae*.

Given the pathogenicity functions attributed to other *avr* genes from *P.syringae*, e.g. *avrE* and *avrRpm1* (Lorang *et al.*, 1994; Ritter and Dangel, 1995), the finding that *avrPphF* was able to restore virulence to compromised plasmid-cured strains such as RW60 was not unexpected. However, *avrPphF* is, to our knowledge, the first gene to be shown to have cultivar- and gene-specific virulence activity. By contrast, the discovery that *avrPphF* also acts as a β *avr* gene, i.e. has functions revealed only in the absence of the PAI, operating in a defined gene-for-gene manner with a matching *R* gene in Canadian Wonder, was most unexpected. The interaction between *avrPphC* and *avrPphF* blocking the HR in cv. Canadian Wonder would appear to occur in the plant, possibly with a receptor for the AvrPphF protein, because *avrPphC* has no effect on the expression of the HR caused by *avrPphF* in cv. Red Mexican. It is important to emphasize that the virulence and β avirulence functions of *avrPphF* are only observed in the absence of other genes from the PAI. The AvrPphF

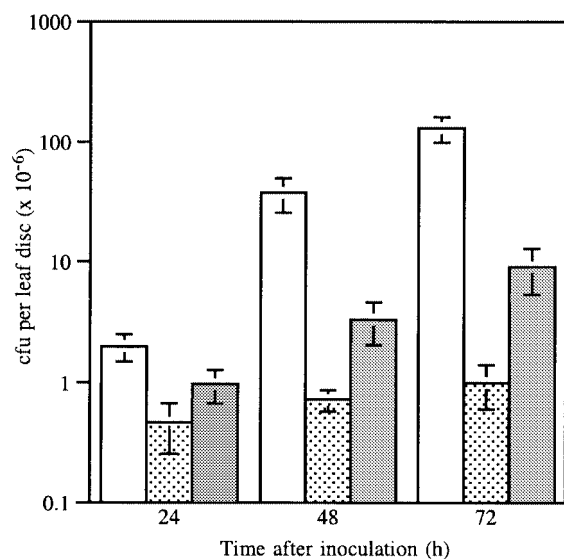


Fig. 7. Bacterial growth in leaves of soybean cv. Osumi inoculated with bacterial suspensions of 10^8 cells/ml of *P.s.* pv. *glycinea* (open), RW60 (stippled) and RW60 (pPPY511) (grey); bars, \pm SEM.

Table V. Genes with dual avirulence and virulence functions, either eliciting or blocking the HR, respectively, identified using strains of *P.s.* pv. *phaseolicola* lacking the 154 kb plasmid which contains the putative pathogenicity island

Gene designation	Plant in which phenotype observed ^a	
	Avirulence	Virulence
<i>avrPphF</i>	Red Mexican ^b , Canadian Wonder ^b	Tendergreen, Soybean
<i>avrPphC</i>	Soybean ^c	Canadian Wonder
<i>virPphA</i> ^d	Soybean	Canadian Wonder, Red Mexican, Tendergreen

^aBean cultivars are named.

^bAvirulence due to interaction with different *R* genes.

^cYucel *et al.* (1994).

^dJackson *et al.* (1999).

Table VI. Bacterial strains and plasmids used in this study^a

Strain/plasmid	Relevant properties	Source or reference
Bacteria. <i>P. syringae</i> pv. <i>phaseolicola</i>		
Principal isolates used		
1375A	race 5, wild-type isolate	D.Teverson ^b
1375AN	race 5, Nal ^R from 1375A	D.Teverson
1375AR	race 5, Rif ^R from 1375A	D.Teverson
1281A	race 1, wild-type isolate	D.Teverson
1281AR	race 1, Rif ^R from 1281A	D.Teverson
1448A	race 6, wild-type isolate	Fillingham <i>et al.</i> (1992)
1448AR	race 6, Rif ^R from 1448A	Fillingham <i>et al.</i> (1992)
1449B	race 7, wild-type isolate	D.Teverson
1449BR	race 7, Rif ^R from 1449B	D.Teverson
RW60	Vir ⁻ , pAV511 ⁻ , Ap ^S , Rif ^R	Jackson <i>et al.</i> (1999)
Additional isolates		
882	race 2	D.Teverson
1301A	race 3	Hitchin <i>et al.</i> (1989)
1302A	race 4	Jenner <i>et al.</i> (1991)
52A	race 5	Hitchin <i>et al.</i> (1989)
2656A	race 8	D.Teverson
2709A	race 9	D.Teverson
<i>P. cichorii</i> 2379	lettuce pathogen	NCPPB ^c
<i>P. s. pv. cornafaciens</i> 1354	oat pathogen	Harper <i>et al.</i> (1987)
<i>P. s. pv. pisi</i> 299A	pea pathogen	J. Taylor ^b
<i>P. s. pv. glycinea</i> 1139	soybean pathogen	this study
<i>P. s. pv. maculicola</i> 1820	brassica pathogen	NCPPB
<i>P. s. pv. tabaci</i> 11528	tobacco pathogen	J. Turner ^d
<i>P. s. pv. tomato</i> DC3000	tomato pathogen	Whalen <i>et al.</i> (1991)
<i>P. s. pv. syringae</i> 281	lilac pathogen	NCPPB
<i>E. coli</i>		
C2110	Nal ^R , <i>polA1</i>	Leong <i>et al.</i> (1982)
DH5 α	Nal ^R , <i>recA</i> , <i>lacZ</i> Δ M15	Bethesda Research Laboratories
HB101	Sm ^R , <i>recA</i>	Boyer and Roulland-Dussoix (1969)
Plasmids		
pBluescript SK ⁺	Ap ^R , multiple cloning sites and priming sites	Stratagene
pHoKmGus	Ap ^R , Km ^R , <i>tnpA</i> , promoterless β -glucuronidase gene in Tn3, pWB15A replicon	Bonas <i>et al.</i> (1989)
pLAFR3	Tc ^R , IncP1 replicon, Tra ⁻ , Mob ⁺ , cosmid	Staskawicz <i>et al.</i> (1987)
pDSK600	Sp ^R , IncQ replicon, 3 \times <i>lac UV5</i> promoter	Murillo <i>et al.</i> (1994)
pRK2013	Km ^R , ColE1 replicon, Tra ⁺ , Mob ⁺ , helper plasmid	Figurski and Helinski (1979)
pSShe	Cm ^R , <i>tnpA</i> ⁺ , pACYC184 replicon	Stachel <i>et al.</i> (1985)
Clones containing <i>avrPphF</i>		
pPPY501	pLAFR3-based genomic clone from race 5 strain 1375	this study
pPPY502	pLAFR3-based genomic clone from race 5 strain 1375	this study
pPPY503	5.3 kb <i>Bam</i> HI– <i>Hind</i> III subclone of pPPY502 harbouring <i>avrPphF</i>	this study
pPPY505	1.8 kb <i>Bam</i> HI fragment from pPPY503 in pBluescript II SK ⁺	this study
pPPY507	1.1 kb <i>Eco</i> RI fragment in pLAFR3	this study
pPPY511	insert as in pPPY505 but in pDSK600	this study
pPPY512	<i>avrPphF</i> ORF2 in pDSK600	this study
pPPY514	<i>avrPphF</i> ORF1 in pDSK600	this study
Additional plasmids		
pMS2330	pLAFR1 based genomic clone harbouring <i>avrPphA</i> from HB33	Shintaku <i>et al.</i> (1989)
pAV511	native plasmid from 1449B, ~154 kb	Jackson <i>et al.</i> (1999)
pAV520	genomic clone harbouring pAV511 sequences in pLAFR3	Jackson <i>et al.</i> (1999)
pAV521	genomic clone harbouring pAV511 sequences in pLAFR3.	Jackson <i>et al.</i> (1999)
pDAHR15	1.4 kb <i>Clal</i> – <i>Bam</i> HI fragment containing <i>avrPphC</i> in pDSK600	Yucel <i>et al.</i> (1994)

^aNal^R, Rif^R, Sm^R, Ap^R, Km^R, Tc^R and Cm^R indicate resistance to nalidixic acid, rifampicin, streptomycin, ampicillin, kanamycin, tetracycline and chloramphenicol, respectively.

^bHorticulture Research International, Wellesbourne, UK.

^cNational Collection of Plant Pathogenic Bacteria, York, UK.

^dUniversity of East of Anglia, Norwich, UK.

proteins appear to block the HR caused by β *avr* genes such as *avr β 1*, which is predicted to be present in the chromosome of *Pph*. The β *avr* function of *avrPphF* is in turn blocked by *avrPphC*. In contrast to *avrPphF*, the other genes recognized to act as virulence determinants, *virPphA* and to a lesser extent ORF4 from the PAI, do not have differential effects in the cultivars tested (Jackson *et al.*, 1999).

The ability of certain Avr proteins to express virulence functions, blocking the phenotype conferred by other *avr* genes, may involve three different types of interaction: (i) between the Avr proteins themselves; (ii) between Avr and masked R proteins; (iii) downstream of hypothetical Avr–R protein interactions to interfere with gene-specific signalling cascades leading to the HR. A possible target for (iii) would be one of the MAP kinases which have been

implicated in plant defence (Grant and Mansfield, 1999; Romeis *et al.*, 1999). The virulence determinant YopJ from *Yersinia* has recently been found to bind and inactivate MAP kinase kinases in mammalian cells, leading to suppression of defence responses (Orth *et al.*, 1999). There are no reports of direct interactions between Avr proteins, but Avr-R protein binding has been demonstrated between AvrPto and Pto (Tang *et al.*, 1996). However, the action of intermediates linking Avr and R proteins has also been proposed (Grant and Mansfield, 1999).

The multiple function of *avr* genes found in *Phaseolus* was also extended to the *Pph*-soybean interaction. Whether or not soybean should continue to be considered as a non-host to *Pph* (Yucel *et al.*, 1994) is questionable as certain strains clearly show weak pathogenicity. Interestingly, however, genes recognized for virulence or avirulence function in *Phaseolus* have so far all displayed the opposite activity in soybean leaves, as summarized in Table V. Pathogenicity towards the legume hosts has probably involved interaction with common virulence targets. As targets mutated to activate resistance (i.e. acting as *R* gene products), perhaps by hyperactivation of signal transduction cascades blocked by Vir factors, the pathogen would need to acquire further *vir* genes whose products would override the activation of resistance. Restoration of virulence could be achieved by suppression of signal transduction, or, as outlined above, simply by blocking any direct *avr*-*R* gene interactions.

An intriguing question is whether or not the *avr* or *vir* genes now identified from a highly evolved pathogen such as *Pph* represent virulence determinants that have had a fundamental role in the evolution to parasitism from a saprophytic ancestry. The layers of interactions and possibly interconnected targets that exist amongst the numerous *avr* and *vir* genes in the present populations of plant pathogens may make it difficult to distinguish the evolutionary significance of individual effector proteins. It is possible that gene-specific virulence, as shown by *avrPphF*, represents a second wave of activity, whereas *virPphA*, apparently with less cultivar specificity, may have more direct effects on signal transduction pathways and represent the first class of virulence factor. The co-evolution of hosts and pathogens would mean that clear distinction between primary and secondary waves of activity may have become very blurred, and it is certainly impossible to predict the function of avirulence and virulence factors we have identified. In order to unravel the complex web of interacting effectors, it is now a priority to identify binding partners in the plant for the Avr proteins recognized to have multiple functions.

Materials and methods

Bacterial strains and plasmids

Principal bacterial strains, cosmids and plasmids used are listed in Table VI. Isolates and transconjugants of *Pph* were grown routinely on King's medium B (KB) agar at 25°C and *E.coli* strains on Luria-Bertani (LB) agar or in LB broth at 37°C (King *et al.*, 1954; Miller, 1972). Antibiotics, obtained from Sigma, were usually used at the following concentrations (µg/ml): rifampicin 60; tetracycline 20; kanamycin 40; ampicillin 100; spectinomycin 40; nalidixic acid 50; chloramphenicol 25.

Pathogenicity tests and in planta bacterial population counts

Pods and leaves of French bean were inoculated as described previously (Harper *et al.*, 1987; Hitchin *et al.*, 1989). The inoculum concentration routinely used in bean leaves was 2×10^8 cells/ml. Soybean plants were inoculated using a 1 ml syringe (without needle) to infiltrate bacterial suspensions of 10^8 cells/ml into the underside of fully expanded primary leaves. Bacterial multiplication in leaves was examined by cutting tissue from the inoculation sites with a 0.6-cm-diameter borer, homogenization in 10 mM MgCl₂, and serial dilution of the homogenate, which was then spread onto LB agar with appropriate antibiotics to allow colony development at 25°C.

General molecular techniques

Basic procedures were carried out as described in Sambrook *et al.* (1989). The methods of Jenner *et al.* (1991) and Mansfield *et al.* (1994) were followed for the construction of the genomic library from race 5 strain 1375A, preparation of minipreps, Southern blotting and hybridization. The library was screened for determinants of avirulence by conjugation of individual clones into race 6 strain 1448AR with the helper plasmid pRK2013. Transconjugants were tested for pathogenicity on pods of cvs Red Mexican and A53 as described by Harper *et al.* (1987). Transposon mutagenesis of pAV521 and pPPY503, and marker exchange of *avrPphF::Tn3gusA33* into race 7 were carried out as described by Mansfield *et al.* (1994); the position of the transposon A33 in the target DNA was confirmed by sequencing.

PCR and DNA sequencing

Standard PCRs were performed with *Taq* polymerase and buffers from Bioline using a Perkin Elmer Gene Amp 2400. Sequencing was performed using the dideoxy chain termination method with the Sequenase enzyme (Pharmacia) as described in the manufacturer's instructions. Primers used for the amplification of *avrPphF* ORF1 were: ORF1F, 5'-ATGAAGAATTCGTTCCGACCG-3'; ORF1R, 5'-TCAGACCGAACTCTCAGACA-3'. For the amplification of *avrPphF* ORF2, the primers used were: ORF2F, 5'-ATGGGTAATATCTGCAATTCG-3'; ORF2R, 5'-GGCCAGTTATAGGAGCTAAT-3'.

Construction of non-polar mutations

For the generation of the non-polar mutants, race 7ΔORF1 and race 7ΔORF2, the following steps were followed. ORF1 was deleted from position 332–439 in pPPY505 by a double digest with *AatII*-*AgeI*. Digested pPPY505 was filled in using Klenow polymerase and re-ligated. Deletions were selected by PCR and the deleted form cloned into pOK (Huguet *et al.*, 1998). ORF2 was deleted from position 767–1157 in pPPY505 by a double digest with *Csp45I*-*Bsr98I*. The plasmid with the digested insert was filled in and re-ligated. Cloned deletions were again selected by PCR and the deleted form was then introduced to pOK. Mutated genes were introduced to race 7 strain 1449BR by homologous recombination in two steps as described by Kaniga *et al.* (1991) and Huguet *et al.* (1998). In both mutants, the presence of the correct deletion in race 7 was verified by PCR.

Acknowledgements

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